

NR2 antibodies: Risk assessment of transient ischemic attack (TIA)/stroke in patients with history of isolated and multiple cerebrovascular events

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ABSTRACT

Background and purpose: Predicting stroke using biomarkers would enable clinicians to help prevent stroke or mitigate damage. Several stroke biomarkers have been investigated but none has shown near term predictive value.

Methods: We studied patients presenting with a history of stroke or transient ischemic attack (TIA) to determine whether serum levels of autoantibodies to the NMDA receptor NR2 peptide (NR2Ab) reflected the presence of recent stroke compared with controls. Antibody levels were also correlated with clinical risk factors for stroke, including diabetes, hypertension, hyperlipidemia, and history of recent TIA or stroke.

Results: Of the 245 patients that presented with acute stroke or TIA, 130 consented to participate and results are available for the 120. Volunteers from the community were recruited as controls. Males and females with multiple recent strokes and females with acute strokes had elevated NR2Ab levels compared to non-stroke patients or controls. Using a multiple regression model, the predictive value for NR2Ab was compared to clinical risk factors. In men, the presence of stroke correlated with hypertension ($p < 0.001$) and NR2Ab levels ($p < 0.01$) and in women the presence of stroke correlated with hypertension ($p < 0.001$), diabetes ($p < 0.05$), atrial fibrillation ($p < 0.05$) and NR2Ab ($p < 0.01$).

Conclusion: These results suggest that NR2Ab levels reflect a history of multiple strokes and may serve as a predictive factor for stroke.

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1. Introduction

During the past decade, several biomarkers for detection of stroke risk and/or acute stroke have been investigated. These include anti-phospholipid antibodies, anti-cardiolipin antibodies, von Willebrand factor, [1,2] C-reactive protein (CRP), phospholipase A2 (PI-PLA₂), [3] matrix metalloproteinase-9 (MMP-9), [4] VCAM, [5] homocysteine, [6] glutamate, neuron-specific enolase, myelin basic protein, S-100b, [7,8] B-type neurotrophic growth factor and monocyte chemoattractant protein-1 [1,2,9].

However, most of these biomarkers have low predictive value for stroke in the near term or reflect the severity of an established subacute or completed stroke. What would be more desirable is a biomarker with near term predictive value. This would be comparable to the stroke-predicting ability of a “crescendo” pattern of transient ischemic attack (TIA). N-methyl-D-aspartate (NMDA) receptor pep-

tides and their autoantibodies (NR2Ab) have been proposed as biomarkers of the neurotoxicity underlying cerebral ischemia and stroke [10]. Anti-NMDA receptor IgG antibodies develop in response to the release of peptide fragments resulting from NMDA receptor turnover during excitotoxicity [10–12], and have been recognized in the serum of stroke patients since the late 1980s [13]. These antibodies were present in the aftermath of stroke and persisted for a period of many months [14]. These antibodies were not present in the serum of patients with Bell's palsy, meningitis or subarachnoid hemorrhage [15].

Preoperative serum concentrations of NR2Ab have been shown to be predictive of severe neurological adverse events after cardiac surgery [16]. Patients with a positive NR2Ab level > 2.0 ng/ml prior to surgery were nearly 18 times more likely to experience a postoperative neurological event than patients with a negative test [16]. The NR2 peptide increases in association with microemboli during carotid endarterectomy and with postoperative neurological deficits following carotid endarterectomy [17,18].

The primary objective of this study was to identify NR2Ab concentrations in patients presenting with a history of multiple transient ischemic attacks (TIAs) or strokes or single or isolated strokes and

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compare these concentrations with controls. A secondary goal was to compare the relative predictive values of NR2Ab and clinical stroke risk factors. We studied patients presenting with acute stroke at DeKalb Medical Center, Decatur, GA, USA. To determine the presence of multiple recent strokes, we reviewed historical, clinical neurological, and neuroimaging data. For controls, we used healthy subjects of different ages without a history of stroke.

2. Materials and methods

2.1. Study design

This was a blinded, convenience cohort study involving a single measurement of NR2 antibody levels in patients with suspected stroke.

2.2. Subjects

Adults aged 18 years or older presenting within 72 h with suspected TIA (defined as a neurological deficit that resolved within 24 h), or suspected acute stroke were included in the study. Patients were excluded if they were pregnant or were transferred to another facility for inpatient care.

Patients were recruited on a convenience basis from January 2006 to January 2008. A number of patients with non-stroke diagnoses were also recruited, as were controls from community patient support groups and meetings of business people. The research protocol was approved by the DeKalb Medical Center Institutional Review Board. Written informed consent was obtained from each subject or a family member.

Blood samples (5 ml) were withdrawn by venipuncture into standard evacuated collection tubes with a serum separator and centrifuged at 4000 RPM for 4 min. Samples were then transferred to 1 ml Eppendorf tubes and frozen at -80°C .

2.2.1. ELISA procedure

Antibody concentrations in the sera were assessed using the Gold Dot NR2 Antibody Test (CIS Biotech, Inc., Atlanta, GA) according to the manufacturer's procedure. Briefly, 100 μl of diluted sera (1:50; 20 μl of serum sample + 980 μl of diluent) and sets of calibrators were added to NR2 peptide-coated wells of microtiter plates and were incubated for 30 min on a shaker at 37°C . After the wells were washed with buffer, 100 μl of Protein A-HRP labeled antibodies was added and incubated for 30 min on a shaker at 37°C . After additional washing, 100 μl of TMB ready-to use substrate was added. The color reaction was developed for 10 min, stopped with stop reagent (100 μl), and measured at 450/630 nm on a microplate reader (ELx800™, Biotek® Instruments, Inc., USA). The NR2Ab titer in each sample was calculated using calibration from the standards provided with Gold Dot NR2 Antibody Test kit.

2.3. Clinical evaluation

All patients had a standard clinical neurological stroke evaluation, including screening for recombinant tissue plasminogen activator (rTPA) when appropriate, standard stroke history, general medical examination and neurological examination. All stroke patients underwent emergent CT scanning and 90% had MRI scans (to be discussed in the later part) performed within 24 h. Each patient was seen and followed during hospitalization by an experienced stroke neurologist. Hospital course and discharge examination data were also noted. Evidence of recent prior strokes was based upon history, available recent records, comparison with previous MRI and CT scans, and the examination of DWI, ADC, T2-weighted and T1-weighted scans for evidence of recent multiple strokes according to the method of Coutts et al. [19]. Clinical determinations were made prior to NR2Ab deter-

minations. All controls completed a standard questionnaire detailing medical history, stroke risk factors, medications, and details of any prior strokes.

2.4. Magnetic resonance imaging and analysis

Images were obtained on a standard clinical MRI scanner (Siemens 1.5 Tesla Avanto) operating with single-shot echo planar-capable gradients. The standard imaging data set comprised T1-weighted sagittal and diffusion-weighted, apparent diffusion coefficient, FLAIR, and T2-weighted axial sequences. Images were reviewed on GE Centricity software.

2.5. Classification of patients and controls

Controls were classified into the "Prior Only" group if they reported a history of stroke. Otherwise, controls were classified in the "No Stroke" group. Patients were classified into one of four groups.

2.5.1. No stroke

Patients admitted with stroke in the differential diagnosis but who had a non-stroke discharge diagnosis or controls without history of stroke. Patients recruited with non-stroke diagnoses were included in this group.

2.5.2. Prior only

Patients admitted with acute stroke in the differential diagnosis but diagnosed with a history of remote stroke or controls with a history of prior stroke or TIA (more than 6 months prior).

2.5.3. Acute only

Patients admitted with acute stroke in the differential diagnosis and discharged with acute stroke as the diagnosis.

2.5.4. Multiple recent

Patients discharged with a diagnosis of acute stroke or TIA and evidence of prior stroke or TIA within 6 months.

2.6. Statistical analyses

Patient data were entered into a Microsoft Access database and exported as spreadsheets. Statistical analysis was performed using R Statistical package (<http://www.r-project.org/>). Standard descriptive statistics were calculated in reporting patient characteristics. A multiple logistic regression analysis, analysis of variance, and Welch two-sample t-test were applied using R.

3. Results

3.1. Patient and control group characteristics

Patient enrollment and disposition are summarized in Fig. 1. The average age, sex distribution, and prevalence of stroke risk factors are shown in Tables 1A and 1B for each of the aforementioned groups. The average age in each of the four groups was 60 ± 5 years.

3.2. NR2 antibody results

Male and female groups were analyzed separately. The mean and standard deviation of NR2Ab levels in each group were determined with descriptive statistics and the results are shown in Tables 1A and 1B. The multiple recent groups had the highest levels of NR2Ab, followed by patients with prior stroke and acute stroke. In all of the female stroke groups, including the no stroke group, NR2Ab levels were higher than in the male groups. The results for each group were compared to the other groups using the Welch two-sample t-test

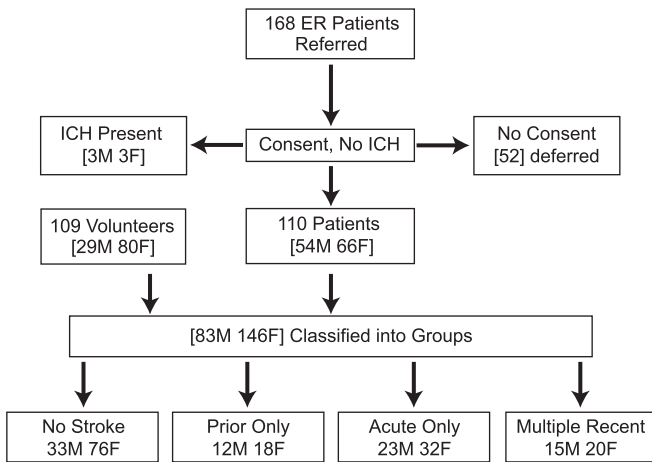


Fig. 1. Disposition of patients enrolled. Abbreviations: ER—Emergency Room. ICH—Intracerebral hemorrhage.

(Tables 2A and 2B). In males, patients with acute stroke but no prior history of stroke had only marginal elevations compared to controls ($p=0.25$), but patients in the multiple recent group had significant elevation compared to the controls ($p<0.05$). In females both acute stroke and multiple stroke groups had significant elevations ($p=0.05$ and $p=0.01$, respectively).

To determine whether substantial differences in NR2Ab levels exist between stroke and non-stroke groups and, therefore whether NR2Ab level could be considered to be the primary predictive variable for stroke, we applied a multiple logistic regression analysis and ANOVA. The data sets for men and women were analyzed separately, both with all data (including statistical outliers) included and with the outliers (as defined in Fig. 2A and B) excluded.

In addition to NR2Ab concentration level, six stroke predictive variables (risk factors) were selected for each individual: hyperten-

Table 2A
Comparison of NR2Ab levels between groups using the Welch two-sample t-test—males (p values).

Group	NR2Ab level ng/ml (SD)	No stroke p value	Prior only p value	Acute only p value
No stroke	1.95 (0.92)			
Prior only	3.09 (1.84)	0.06		
Acute only	2.43 (1.84)	0.25	0.32	
Multiple recent	4.15(3.5)	<0.05	0.32	0.10

Table 2B
Comparison of NR2Ab levels between groups using the Welch two-sample t-test—females (p values).

Group	NR2Ab level ng/ml (SD)	No stroke p value	Prior only p value	Acute only p value
No stroke	2.05 (1.81)			
Prior only	3.72 (2.69)	0.06		
Acute only	2.50 (1.48)	<0.05	0.09	
Multiple recent	4.00 (3.59)	<0.01	0.79	0.09

sion, diabetes, atrial fibrillation, heart disease, remote (more than 6 months prior) TIA, and history of elevated cholesterol.

In the males, with all groups combined, tests conducted for the fitted regression model with eight variables indicated that only two out of eight variables, NR2Ab level and history of hypertension, were statistically significant predictors for stroke.

When the fitting of the full regression model with two predictors was performed, no significant interactions between predictors were found. The interpretation of the estimated regression coefficients is that males in our group have odds in favor of having a stroke that was increased by two factors: 2.5-fold with a unit increase of NR2Ab level, and more than 64-fold with hypertension (Table 3A).

Table 1A
NR2Ab levels and frequency of disease based on patient histories—males.

	N	Age, yr (mean)	NR2Ab ng/ml (SD)	Hypertension	Diabetes mellitus	Atrial fibrillation	Elevated cholesterol	Heart disease
Total	81	61.6	2.66 (2.13)	50	23	7	23	14
No stroke	31	62.4	1.95 (0.92)	9	6	2	12	4
Prior only	12	65.3	3.09 (1.84)	11	7	2	4	2
Acute only	23	57.4	2.43 (1.84)	17	6	2	2	6
Multiple recent	15	63.5	4.15 (3.50)	13	4	1	5	2

Table 1B
NR2Ab levels and frequency of disease based on patient histories—females.

	N	Age, years (average)	NR2Ab ng/ml (SD)	Hypertension	Diabetes mellitus	Atrial fibrillation	Elevated cholesterol	Heart disease
Total	107	63.7	2.83 (2.42)	66	25	13	37	13
No stroke	37	63.6	2.05 (1.81)	11	3	1	18	2
Prior only	18	68.3	3.72 (2.70)	13	7	3	3	2
Acute only	32	65.5	2.50 (1.49)	26	9	6	10	6
Multiple recent	20	57.0	4.00 (3.60)	16	6	3	6	3

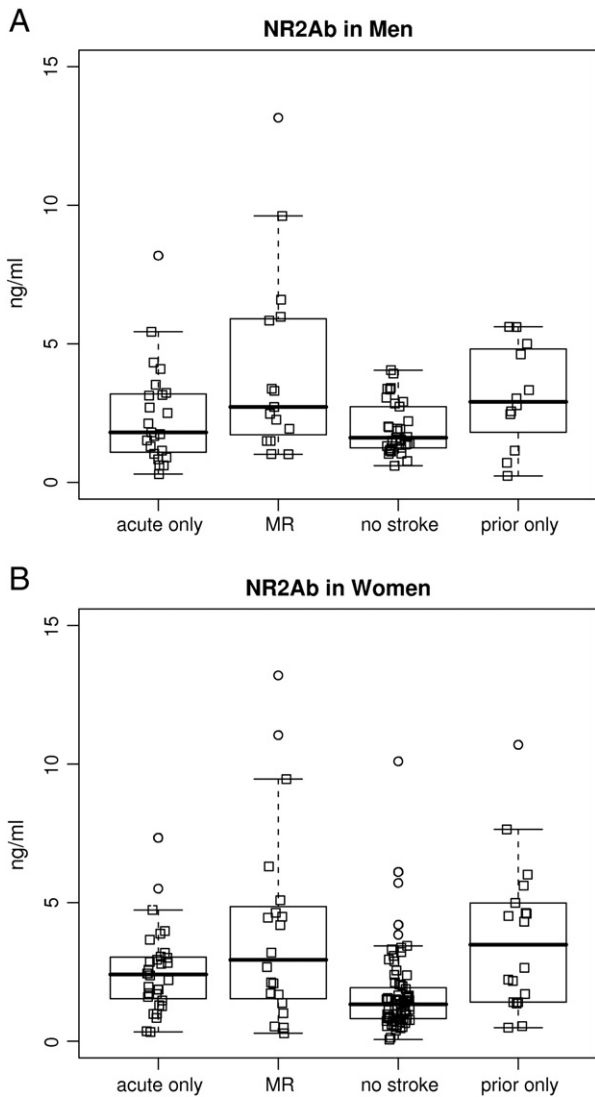


Fig. 2. A. NR2 antibodies in males. The y-axis represents antibody concentration in pg/ml. The x-axis represents the different subject groups as outlined in the [Materials and methods](#) section. In these box and whisker plots the following convention is used: the bottom and top of the box are the 25th and 75th percentiles and the thick central line is the 50th percentile (median). The lower whisker intersects the lowest datum still within 1.5 times the distance between the 25th and 50th percentiles and the upper whisker intersects the highest datum still within 1.5 times the distance between the 50th and 75th percentiles. Circles are used to represent data that lie outside the whiskers; these might be considered outliers. B. NR2 antibodies in females. The y-axis represents antibody concentration in pg/ml. The x-axis represents the different subject groups as outlined in the [Materials and methods](#) section. In these box and whisker plots the following convention is used: the bottom and top of the box are the 25th and 75th percentiles and the thick central line is the 50th percentile (median). The lower whisker intersects the lowest datum still within 1.5 times the distance between the 25th and 50th percentiles and the upper whisker intersects the highest datum still within 1.5 times the distance between the 50th and 75th percentiles. Circles are used to represent data that lie outside the whiskers; these might be considered outliers.

In females, with all subject groups combined, the generalized linear regression model identified four significant predictive variables, which were confirmed using ANOVA analysis: NR2Ab levels, diabetes, hypertension, and presence of atrial fibrillation ([Table 3B](#)). The interpretation of the estimated regression coefficients in this model is that females in our group had odds in favor of having a stroke that was increased by a factor of 2.5-fold with each unit increase of NR2Ab level; 3.25-fold with diabetes; 7.4-fold with hypertension; and more than 11.8-fold with atrial fibrillation ([Table 3B](#)).

Table 3A
Predictive variables for stroke: males.

Parameter	P value		
	Without outliers	Without outliers, With age/TIA	With outliers
Age		0.93	
TIA		0.99	
NR2Ab	<0.01	<0.01	<0.01
Diabetes	0.24	0.23	0.94
Hypertension	<0.001	<0.001	<0.001
Atrial fibrillation	0.95	0.80	0.99
Heart disease	0.94	0.75	0.72
Cholesterol	0.86	0.70	0.65

Overall, for both male and female groups, these conclusions were not changed when deleted outliers were included in the calculations ([Tables 3A and 3B](#)).

4. Discussion

Biomarkers offer the possibility for both the acute detection of stroke and the estimation of stroke risk. These are areas that continue to present a clinical challenge in complicated situations. Examples would include patients with prior strokes, fluctuating strokes, and concomitant disorders, such as multiple sclerosis. In these situations, current diagnostic tests, including MRI with or without diffusion-weighted imaging, may give inconclusive information. Also, the transient ischemia that presumably underlies “true” TIAs may not be detectable *post facto* by current methods. A biomarker that would reflect the early phase of neuronal excitotoxicity and ischemic dysfunction might provide additional diagnostic information about “true” TIAs.

The ideal biomarker for acute detection of ischemia and stroke has yet to be identified. Neuron-specific biomarkers have been studied, but these are predominantly derived from the cytoplasm and cytoskeleton of neurons [20] and presumably reflect end-stage necrosis rather than early stage excitotoxicity. As it is well known, the activation of the excitotoxicity cascade results early on in the activation of cell surface excitatory amino receptors and the release of polypeptides from these receptors by the action of endogenous proteases, a process that does not require cell death.

The N-methyl-D-aspartate receptor (NMDAR), is one of the key regulators of the transduction of neuronal electrical signals and plays an important role in excitotoxicity in part by its permeability to both sodium and calcium ions. It is well known that NMDA receptors are localized on both pre- and post-synaptic surfaces of neurons but Sharp et al. [21] have recently shown that these receptors are also present on the epithelial surface of cerebral microvessels, that form the blood-brain barrier and control microvessel function. Furthermore, activation of NMDA receptors on neuroepithelial cells by L-glutamate results

Table 3B
Predictive variables for stroke: females.

Group	P value		
	Without outliers	Without outliers, With age/TIA	With outliers
Age		0.68	
TIA		0.99	
NR2Ab	<0.01	<0.01	<0.001
Diabetes	<0.05	0.07	0.06
Hypertension	<0.001	<0.01	<0.001
Atrial fibrillation	<0.05	<0.05	<0.05
Heart disease	0.72	0.80	0.57
Cholesterol	0.34	0.41	0.21

in decreased integrity of the endothelial barrier [22]. Very recent work by Betzen et al. [23] also suggests that oxidative stress up-regulates the amount of NMDA receptors on cerebral blood vessels. Thus a population of NMDA receptors is outside the blood-brain barrier, is in contact with the blood circulation, and is up-regulated by oxidative stress.

NMDA receptors not only mediate excitotoxicity as in the case of cerebral ischemia, but also undergo modification themselves. In the early phases of ischemia, thrombin-activated serine proteases are activated and release fragments (termed NR2 peptides) from the NMDA receptor into those which are released through a compromised blood-brain barrier into the blood stream where they can be measured [24]. Antibodies to NMDA receptor peptides (NR2Abs) develop in response to the release of NR2 peptide fragments and can be measured in the blood [17].

The prediction of stroke risk is another area that continues to develop. Most research to date with biomarkers has concentrated on near term prediction of stroke progression or hemorrhagic transformation [25,37]. Biomarkers would have useful application in the challenge of determining which patients are at greater risk for near term stroke. This would have application in establishing the effectiveness of treatments in primary stroke prevention.

Most preventive therapy for stroke has concentrated on secondary prevention of stroke. The greater progress in this area reflects the relatively greater risk of stroke recurrence after an initial identified stroke compared to stroke-free individuals with risk factors such as diabetes, hypertension, or hyperlipidemia. Even in patients with atrial fibrillation, prior stroke or TIA has the greatest predictive value among the “clinical” biomarkers [26].

Recurrent “crescendo” TIA is also a predictor of near term stroke risk in carotid stenosis [27]. Given that the ratio of asymptomatic stroke or subclinical strokes has been estimated as high as 5:1 on the basis of MRI [28], it is likely that most patients with a clinical stroke have been confronted with at least one subclinical event. A biomarker that would reflect either subclinical events or TIA might be as useful, or more useful, than measures of degenerative or inflammatory changes in carotid artery plaques.

Recent studies in patients undergoing carotid artery endarterectomy surgery and carotid artery stenting surgery for carotid stenosis have shown that NR2 peptide levels were more elevated in patients with a recent history of symptomatic carotid stenosis. Also, transcranial Doppler measurements of embolic particles during endarterectomy or stenting correlated with neurological adverse events postoperatively [18].

4.1. Effect of multiple recent events on NR2Ab levels

The NR2Ab directed to the N-terminal peptide fragment of the NMDA receptor develops following an initial event, where NR2 peptides are released in association with excitotoxicity. Unlike the peptide, the antibody would not be expected to appear acutely, but would require a delay due to immune system antigen recognition. Given that most strokes are clinically silent, it is likely that patients presenting with a first stroke would have had prior exposure to the NR2 peptide, thus accounting for the appearance of antibodies to these peptides following a first clinical stroke. The NR2Ab may have similar predictive value for a recent TIA or stroke or for the prediction of the next stroke. To the extent that the NR2Ab biomarker declines in concentration after an initial stroke, it may also serve as a marker for distinguishing between subacute and remote strokes.

Our results demonstrate that the NR2Abs are present in higher level in patients with multiple recent vascular events compared to first or isolated events. This is compatible with the mechanism of NR2Ab production, which results from immunization of the host upon release of NR2 peptide fragments. There may be a summation effect from multiple recent ischemic events. This finding is similar to the

recent work of Coutts et al. [19], in which patients presenting with acute stroke who had additional imaging findings of prior (subacute) asymptomatic stroke on DWI/MRI were found to be at greater risk for future stroke.

4.2. Subclinical and reversible ischemic events and NR2Abs

Acute stroke may be frequently asymptomatic and produce no symptoms or deficits. Studies of CT and MRI scans obtained from patients presenting with their first acute cerebral ischemic event have shown that patients often (14%–28%) have evidence of prior silent infarcts [29,30]. This percentage is likely higher when MRI data are used. However, cerebral infarction may present clinically as a TIA [31], presumably because rapid cortical compensation or reorganization effectively compensates for the neurological deficit.

An indication of the potential for reversible ischemia to activate excitotoxic processes and produce NMDA receptor fragments occurred recently at our institution. An elderly patient with carotid stenosis and no prior history of completed stroke presented to the hospital with a one-month history of multiple recurrent transient ischemic attacks producing transient right hemiparesis. A blood sample obtained during her initial presentation with such an episode detected elevated levels of NR2Abs and NR2 peptides. A non-contrasted head CT scan was negative. The hemiparesis subsequently cleared completely, with no detectable deficit though the patient did ultimately experience a stroke. This observation, however limited as a single case report, strongly suggests that NMDA receptor peptides reflect acute and potentially reversible ischemia. NMDA receptor autoantibodies reflect a history of ischemia including transient reversible ischemia, though they are not released acutely.

4.3. NR2Abs in prediction/risk assessment of TIA and stroke

This study provides several important findings. First, patients with stroke had elevated blood levels of NR2Ab compared to stroke-free controls. Second, NR2Ab levels were significantly elevated in patients with multiple versus isolated events. Finally, multivariate analyses found NR2Ab levels and history of hypertension were statistically predictive for stroke in males and NR2Ab levels and history of diabetes, hypertension and atrial fibrillation were predictive of stroke in females. In addition, NR2Ab levels were elevated in female subjects compared to males with higher levels of antibodies in some asymptomatic women. This latter finding may possibly relate to the greater prevalence of silent brain infarcts and leukoaraiosis in women that have been observed in several surveys of patients with first-ever ischemic stroke [32] and in population-based studies [33,34]. This may also relate to sex-dependent differences in the immune response. Recent work by Niehusman et al. [35] on a related but distinct epitope of the anti-NMDA receptor antibody first identified in paraneoplastic disorders associated with ovarian teratomas, suggests that certain anti-NMDA antibodies to paraneoplastic-associated epitopes may have an increased prevalence in women, justifying a sex-specific analysis. The finding that atrial fibrillation was predictive of stroke in the females in our study but not in the men may be accounted for by the observation of Fang et al. [36] regarding the twice greater risk of uncontrolled atrial fibrillation in women versus men. Our relatively small sample size may not have had the statistical power to detect the predictive effect of atrial fibrillation for stroke in the males.

The ANOVA analyses that we performed demonstrated a statistically significant correlation of NR2Ab levels with the stroke state in both male and female groups, supporting that NR2Ab level could serve as a predictive factor for stroke.

We conclude that NR2Ab level is a predictor factor or biomarker for stroke. Compared to typical risk factors such as diabetes, hypertension, hyperlipidemia, and atrial fibrillation, a biomarker such as the NR2Ab level is quantitative and does not depend on patient

recall, compliance, or moment-to-moment variability, as in the case of nocturnal blood pressure elevations.

There are several limitations to the present study. Patient numbers are small, and resources required recruitment on a convenience basis. We relied on patients and controls reporting risk factors and other elements of stroke risk-related history, as old records were not always available. In addition, it was not feasible to evaluate control subjects with MRI for the presence of prior asymptomatic stroke.

Despite these limitations, our results demonstrated that NR2 antibodies are associated with stroke risk and appear to be increased in the setting of multiple recent strokes. Anecdotal evidence suggests that reversible ischemia may produce detectable changes in NR2 peptides and NR2Abs. A blood assay detecting NR2Ab could be a beneficial, low-cost aid to clinical observations in the recognition of impending stroke, particularly if symptoms of cerebral ischemia are subtle or asymptomatic.

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